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This combined report of research and career development grants entails three objectives. Progress on the first objective this year centered on an analysis of the importance of a functionally intact TGF-β signaling pathway with regards to tumor necrosis factor (TNF)-mediated cytotoxicity of MCF-7 cells. We have documented that loss of TGF-β responsiveness resulted in increased Bcl-2 levels and marked resistance to TNF. The second objective of the grant was to evaluate the in vivo tumorigenicity and metastatic potential of breast cancer cells altered with regard to their production of and responsiveness to TGF-β. Those experiments are still underway, and no conclusion has been reached. The third onjective was to identify the molecular determinants of promoter usage for TGF-β3 in breast cancer cell lines. In the past year we have observed that whereas CpG sites closest to the breast cancer-specific downstream promoter are nearly completely methylated in non breast cancer cells, they are mostly unmethylated in breast cancer cells. This is consistent with the observed expression pattern of these cells, and suggests that CpG methylation is the basis of this difference.

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Bradley Carter 10/29/98

PI - Signature Date

ANNUAL REPORT
Grant Numbers DAMD17-J-4287 & DAMD17-J-4130
PI: Bradley A. Arrick, M.D., Ph.D.
Institution: Dartmouth College
Reporting Period: 10/1/97-9/30/98

Titles:

Analysis of the Regulation of Expression of Transforming Growth Factor-Beta in Human Breast Cancer Cells (#4287)

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Studies on Human Breast Cancer and Transforming Growth Factor-beta -- Application for a Career Development Award (#4130)

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Studies on Human Breast Cancer and Transforming Growth Factor-beta -- Application for a Career Development Award (#4130)

Introduction

The above-listed grants were the focus of a USAMRMC Breast Cancer Research Project Site Visit, conducted on the campus of Dartmouth College on December 4, 1997. The site visit panel's final recommendations included that I modify the Statement of Work to reflect some of the changes in scientific focus that have occurred during the course of this research, merge the Statements of Work for the two grants into one (since one of the grants is a career development award funding partial effort on some of the same research), apply for a no-cost extension, and consolidate my annual report for the two grants into one. This past summer I applied for, and received, a one-year no cost extension (letter to Michael A. Younkins, dated July 24, 1998). At that time I provided a revised Statement of Work, placing the current and planned research into three objectives. This report is therefore a combined report, based on that revised SOW.

Body of Report

Objective #1

Background. In last year's report, we provided data which refutes earlier claims that high expression of TGF-β1 by breast cancers portends a poor clinical outcome. We have also found that few if any breast cancers (<20%) have gene amplification of the TGF-β1 locus. These data were discussed in length at the site visit, and as described on page 3 of the site visit report, we have replaced the remainder of Objective #1 with an analysis of an apparent dependence upon TGF-β signaling for growth suppression by tumor necrosis factor-α (TNF-α) in breast cancer cells. This stemmed from a growing appreciation that many tumors become unresponsive to the effects of TGF-β at some critical stage in malignant progression. This raises the question: which aspects of TGF-β's many effects on cells are important in this regard? We turned our attention to the proposed role for TGF-β in regulating apoptosis. Apoptosis, or programmed cell death is a specific form of cell death which is characterized by discreet morphologic and bio-chemical events. Many groups have documented an increase in TGF-β message and/or protein as an early event in the apoptotic cascade. Examples include removal of hormone from hormonally-dependent cells (Kyprianou et al., 1991), antimetabolite

induced apoptosis of hormone-independent cells (Armstrong et al., 1992), and treatment of human mammary carcinomas with pharmacological doses of antiestrogens (Huovinen et al., 1993; Warri et al., 1993). What remains unclear, however, is whether the observed rise in TGF-β was an integral event of the apoptotic cascade or whether it was in fact a consequence of the cascade.

One of the most recently published investigations into the role of TGF-β as a potential mediator of cell killing involves the cytotoxic effect of TNF-α. TNF-α is a cytokine capable of binding and activating the cell surface receptor TNF-RI. TNF-RI is a member of a family of death inducing receptors which includes Fas/CD95 and DR3 (death receptor 3). Binding of TNF-α to TNF-RI is responsible for the generation of most of the known cellular responses of TNF-α, including apoptosis and activation of NF-κB. Danforth and Sgagias observed that breast cancer cell lines that were susceptible to TNF-α exhibited an increase in TGF-β production within 24 hrs after exposure to TNF-α and prior to cell death (Danforth and Sgagias, 1996). They tested the hypothesis that the induction of TGF-β, occurring before cytotoxicity was evident, was required for TNF-α-mediated killing by co-incubation with neutralizing anti-TGF-β antibody. This had no effect on the degree of cytotoxicity in their experiments, leading to the conclusion that TGF-β played no critical role in TNF-α–mediated growth inhibition of MCF-7 cells.

Such a conclusion, based on the exogenous addition of neutralizing antibody, cannot be considered a definitive assessment of the role of the TGF-β pathway in this process since exogenous antibody may not gain access to all the TGF-β capable of eliciting an effect. Feeling that a TGF-β nonresponsive breast cancer cell line could be useful to re-examine more conclusively the observations of Danforth and Sgagias (and later investigation into other aspects of TGF-β signaling in these cells), we transfected MCF-7 cells which are innately sensitive to both TGF-β and TNF-α with a truncated TGF-β type II receptor (TβR-II). Others have demonstrated that when overexpressed, a truncated TβR-II acts as a dominant negative mutation resulting in loss of TGF-β responsiveness (Brand and Schneider, 1995). Comparison of the resulting TGF-β nonresponsive cells with their TGF-β responsive counterparts with regards to their susceptibility to TNF-α induced cell death allowed us to directly determine if the increased cellular production of TGF-β is an integral participant in the cascade leading to apoptosis.

In the past year, work has proceeded on the following sub-aims:

- Generation and initial characterization of TGF-β-responsive and TGF-β-nonresponsive subclones of MCF-7 cells.
- Dose-response analysis to calculate the IC_{50} of TNF for TGF-β-responsive vs. TGF-β-nonresponsive MCF-7 cells.
- Comparison of the expression of TNF receptors and downstream mediators of TNF action in TGF-β-responsive vs. TGF-β-nonresponsive MCF-7 cells.
- Comparison of the expression of regulators of apoptosis (including bcl-2) in TGF-β-responsive vs. TGF-β-nonresponsive MCF-7 cells.

Results

Generation and initial characterization of TGF-β-responsive and TGF-β-nonresponsive subclones of MCF-7 cells.

In order to investigate the role of TGF-β signaling in apoptosis of breast cancer cells, TGF-β responsive MCF-7 cells were transfected with a truncated TβR-II to create TGF-β-nonresponsive sublines/counterparts. The mammalian expression plasmid used ('TREZ') codes for a bicistronic message under the control of the CMV promoter. The resulting bicistronic mRNA contains a C-terminally truncated TβR-II sequence which lacks the cytoplasmic kinase domain followed by IRES sequence which is followed by zeocin resistance as a selectable marker. MCF-7 cells transfected with the TREZ plasmid or the EZ empty control plasmid were grown in the presence of 50 ng/ml zeocin for several weeks and zeocin resistant clones were isolated. These stable transfectants were screened by western analysis to verify expression of the truncated TβR-II (Fig. 1).

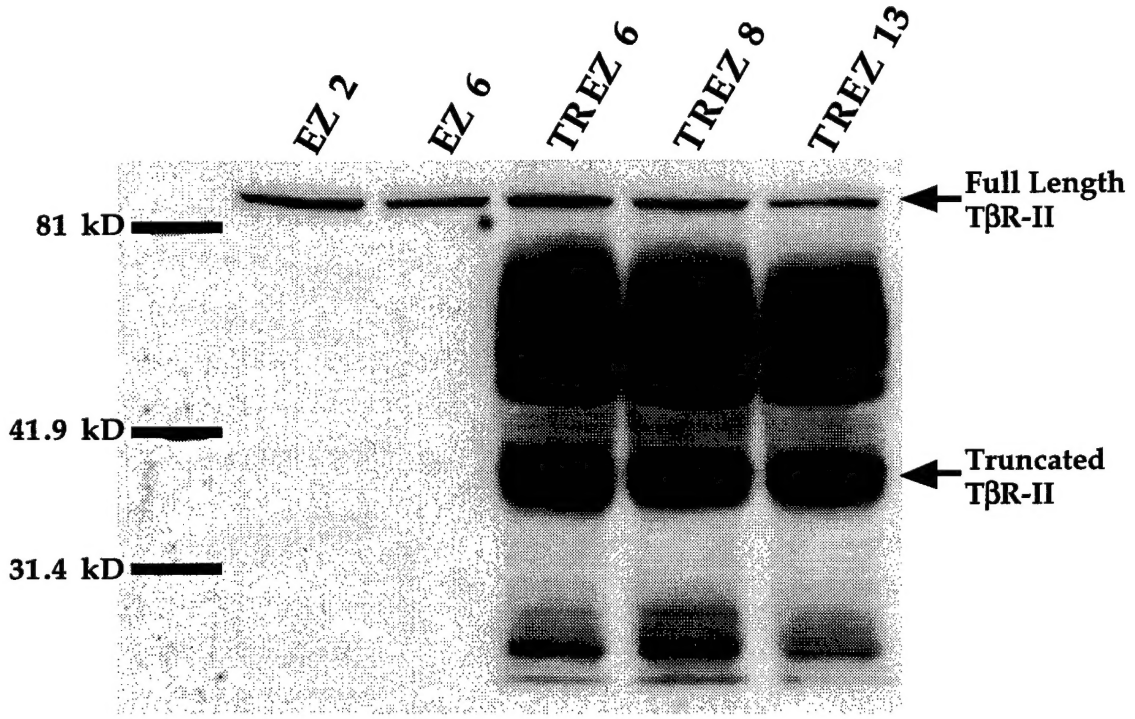

Figure 1. Expression of full length endogenous and plasmid-encoded truncated TβR-II in MCF-7 stable transfectant clones. Cell lysates were prepared and analyzed by western blot using standard procedures. Each lane contains 50 μg of total protein and the primary antibody used was anti-TGF-β type II receptor (L-21 from Santa Cruz). The western blots were developed by chemiluminescence and exposed to film. The epitope of the L-21 antibody consists of amino acids 246-266 and is present in both the truncated and endogenous receptors. Note that all clones express the full length receptor near 85 kD and only the TREZ clones express the truncated receptor near 38 kD. The intermediate bands are believed to be aggregates of the overexpressed truncated receptor.

As expected, the TREZ clones express high levels of truncated TβR-II in addition to the full-length endogenous TβR-II while the EZ clones express only the full-length endogenous receptor. The TβR-II bands of intermediate size found in the TREZ clones are most likely multimerization of this highly overexpressed transmembrane protein. It should be noted that the expression level of the truncated TβR-II in TREZ clones is many fold higher than that of the endogenous receptor, suggesting that an effective dominant negative effect might be evident in these cells.

TGF-β's growth inhibition of epithelial cells provides a convenient and accepted assay for measuring TGF-β sensitivity. To functionally determine the TGF-β responsiveness of the MCF-7/TREZ and the MCF-7/EZ stable clones, cell proliferation of each clone was measured in the presence or absence of TGF-β (3 ng/ml) for 5 days (Fig. 2).

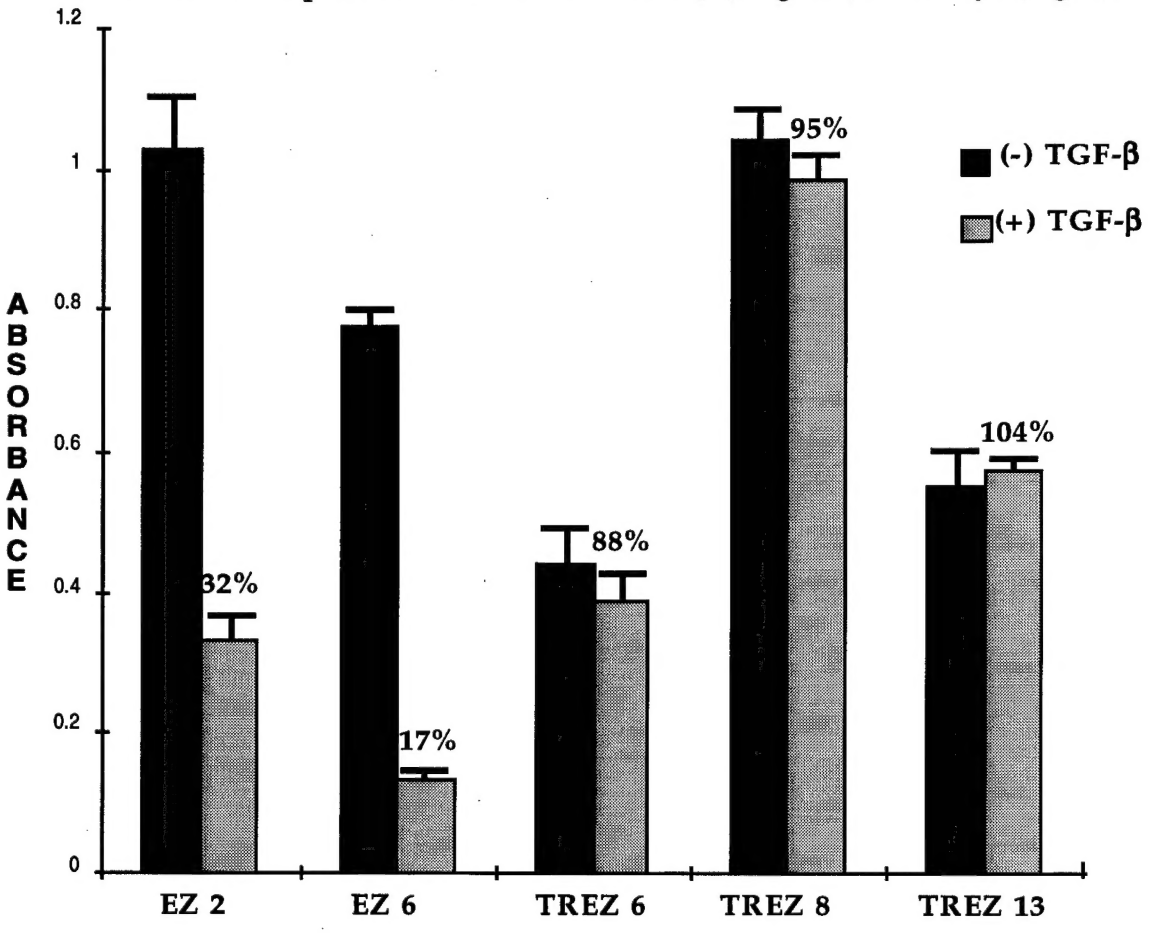

Fig. 2. Truncated TβR-II functions as a dominant negative mutation and effectively blocks TGF-β signaling. Cells were counted and plated in triplicate in a 96-well plate at 3000 cells/well. TGF-β was added to triplicate wells at doses of 0 or 3.0 ng/ml and cells were placed in the 37°, 5% CO_2 incubator for 5 days. A colorimetric MTS reduction assay (Promega) was conducted to determine the relative number of viable cells. Above each bar for the TGF-β treated cells, for each clone, is the calculated percentage of their untreated control cells.

In parallel dose-response experiments, we have determined that MCF-7/EZ clones retain their TGF-β responsiveness and display 50% growth inhibition by TGF-β doses <1ng/ml, while the MCF-7/TREZ clones showed no growth inhibitory effect out to doses of 3ng/ml of TGF-β. The loss of TGF-β sensitivity in the MCF-7/TREZ clones indicate that the truncated TβR-II functions as a dominant negative inhibitor of TGF-β signaling in these cells.

In order to evaluate whether or not TGF-β signaling plays a functional role in TNF-α induced cytotoxicity, TGF-β responsive MCF-7/EZ and TGF-β nonresponsive MCF-7/TREZ clones were treated with a wide range of TNF-α concentrations for 5 days (Fig. 3). The TGF-β responsive MCF-7/EZ clones were highly sensitive to TNF-α's cytotoxic effects characterized by an IC_{50} of 100 U/ml. The TGF-β nonresponsive MCF-7/TREZ clones were markedly resistant to TNF-α induced cell death and displayed an IC_{50} of 800 U/ml. This eight fold increase in IC_{50} indicates that TGF-β signaling via an autocrine loop does play a functional role in (enhances) TNF-α induced cytotoxicity.

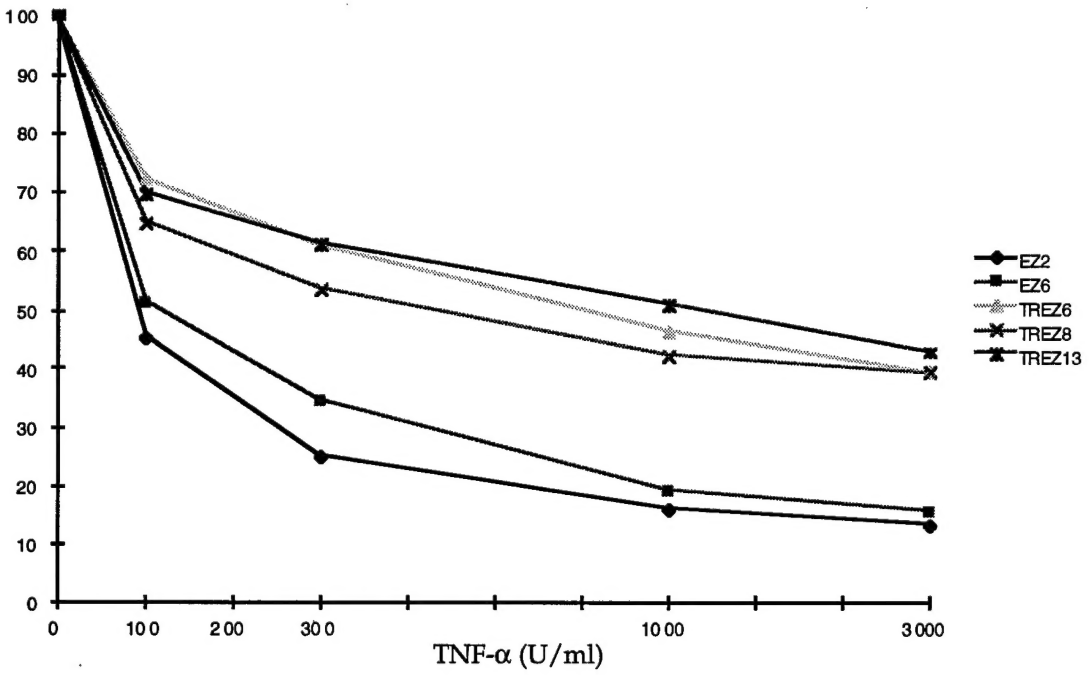

Fig. 3. TGF-β signaling plays an active role in TNF-α induced cytotoxicity. Cells were counted and plated in triplicate in a 96-well plate at 3000 cells/well. TNF-α was added to triplicate wells at doses ranging from 0 to 3000 U/ml and the cells were placed in a 37°, 5% CO_2 incubator for 5 days. A colorimetric MTS reduction assay (Promega) was conducted to determine the relative number of viable cells in each well. Values given are percent of untreated control. Note that the X-axis is non-linear between 300 and 1000 U/ml TNF-α. Y-axis is percent of untreated control for each clone. The TGF-β responsive MCF-7/EZ clones show an average IC_{50} of 100 U/ml TNF-α, while the TGF-β nonresponsive MCF-7/TREZ clones have an average IC_{50} of 800 U/ml.

On a molecular level, the TNF-α cell death pathway starts when TNF-α ligand binds its receptor TNF-RI (p55) resulting in recruitment of TRADD (TNF Receptor Associated Death Domain protein) to the cytosolic death domain of the receptor. To assess whether or not TGF-β signaling affects the expression level of these two proteins critical to the early steps of TNF-α induced cytotoxicity, western analysis was conducted on cell lysates of TGF-β responsive MCF-7/EZ clones and TGF-β nonresponsive MCF-7/TREZ clones (Fig. 4). Figure 4A shows that equal levels TNF-RI receptor is expressed in all clones and figure 4B shows that TRADD expression levels are almost equal. These data argue that TGF-β signaling has not altered the expression of these two proteins and that the marked difference between the MCF-7/TREZ and the MCF-7/EZ clones in their sensitivity to TNF-α induced cytotoxicity observed in Figure 3 cannot be attributed to changes in TNF-RI or TRADD protein expression.

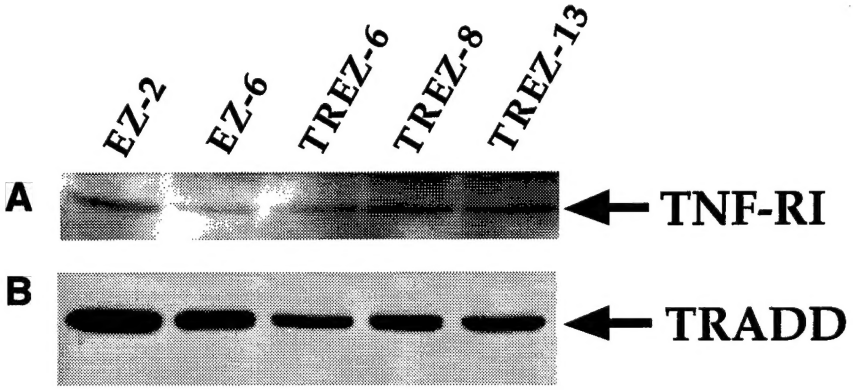

Fig. 4. TGF-β does not affect expression of TNF-RI (p55) or TRADD. Cell lysates were prepared and 100 μg of protein was loaded per lane of an SDS-PAGE gel. Western immunoblot analyses were carried out using (A) anti-TNF-RI or (B) anti-TRADD as the primary antibody.

Evidence in the literature has established that TGF-β treatment can alter the activation of the transcription factor NF-κB in certain systems. Moreover, it has been shown that activation of NF-κB can provide an anti-apoptotic signal thereby promoting cell survival. In order to determine if TGF-β is affecting NF-κB activity in MCF-7 cells resulting in altered sensitivity to TNF-α induced cell death, NF-κB activity in MCF-7/EZ and MCF-7/TREZ clones was measured following treatment with 10,000 U/ml of TNF-α by gel shift assay (Fig. 5). The levels of NF-κB activation in response to TNF-α are equivalent in both TGF-β responsive and nonresponsive clones and the maximum activity appears to be reached within the first hour of treatment. This result indicates that TGF-β's effect on TNF-α induced cytotoxicity is not mediated by changes in NF-kB activity. Additionally, these data point out that although the TGF-β nonresponsive MCF-7/TREZ clones are resistant to the cytotoxic effects of TNF-α, these cells are still capable of responding to TNF-α ligand and that certain branches of

the TNF-α signaling cascade remain as sensitive as is found in the TGF-β responsive MCF-7/EZ controls.

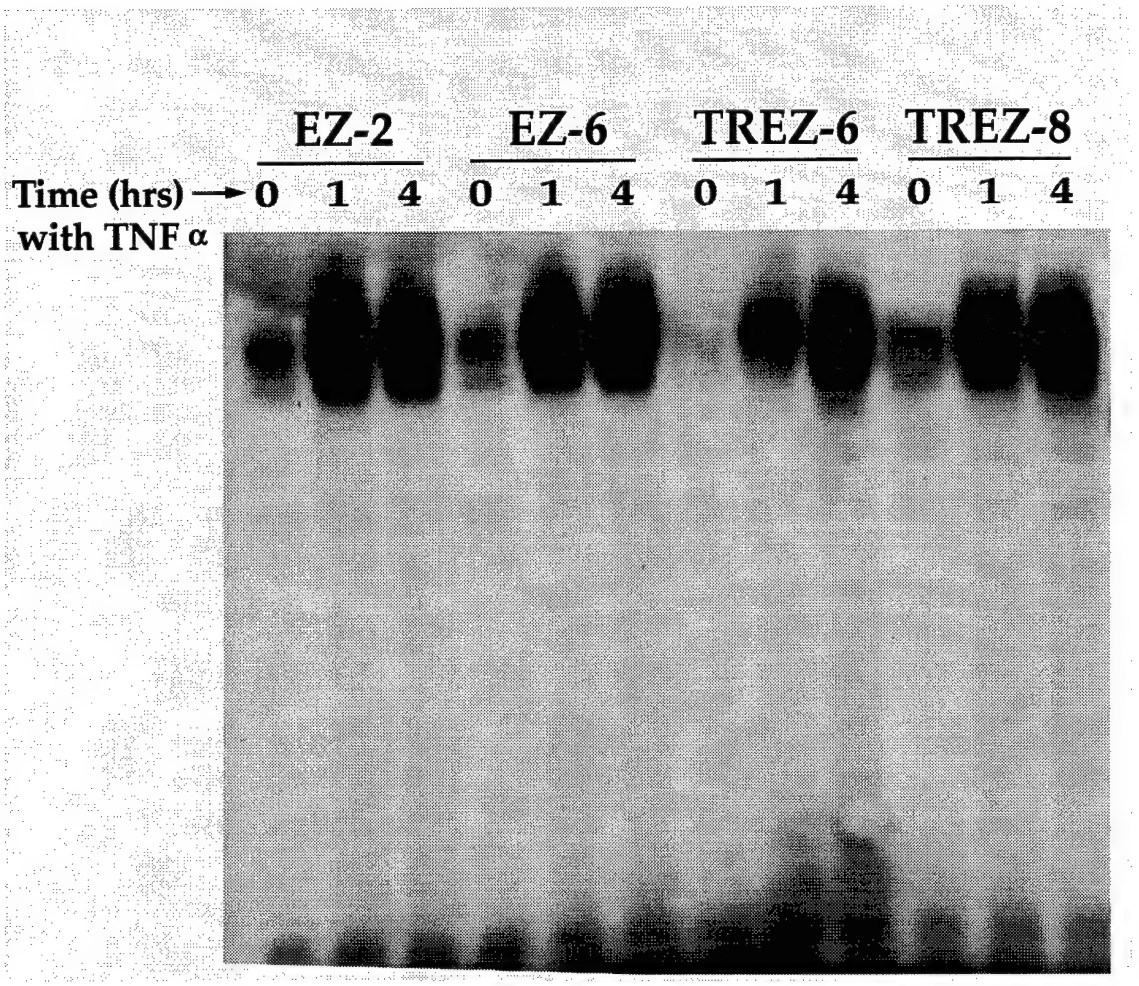

Fig. 5. Autocrine TGF-β signaling does not alter basal levels or TNF-α induced levels of NF-κB activity. Cells were plated in 6-well plates and on the following day were treated with or without TNF-α at 10,000 U/ml. Treatment times were 1 hour and 4 hours. Activation of NF-kB by TNF-α treatment was measured by gel shift assay. Nuclear extracts were harvested and 2 μg of nuclear protein was incubated with 200,000 cpm of a ^{32}P end labeled probe. The probe was prepared with a dsDNA oligo containing the consensus kB binding sequence. Following the 20 minute incubation, the samples were run on a non-denaturing 4% polyacrylamide gel, the gel dried and exposed to film. Note: The MCF-7/TREZ-6 sample treated for one hour (lane 8) had only approximately 0.4 μg of nuclear protein.

It has been previously shown that TGF-β signaling can regulate the expression of Bcl-2 family members and that the Bcl-2 family of proteins is a key regulator of cell death induced by many stimuli, including TNF-α (REFHERE). In order to ascertain whether or not TGF-β is altering the cell's resistance to TNF-α induced cell death by modulating the expression of Bcl-2 family members, western analysis was conducted on cell lysates of TGF-β responsive MCF-7/EZ clones and TGF-β nonresponsive MCF-7/TREZ clones (Fig. 6).

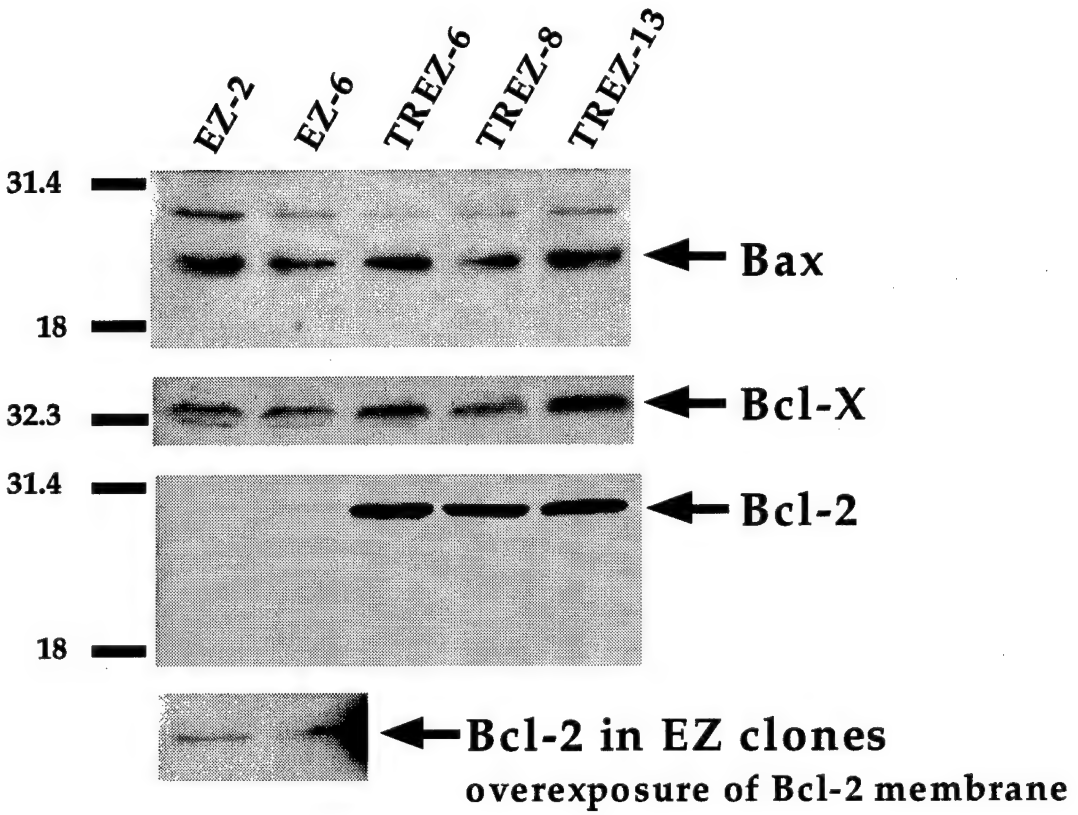

Fig. 6. Bcl-2 expression is downregulated in MCF-7/EZ clones when compared to MCF-7/TREZ clones. Cell lysates were prepared and 100 μg of protein loaded per lane of an SDS-PAGE gel. Western immunoblot analyses were carried out using primary antibodies to various members of the Bcl-2 family of proteins. The western blots were developed by chemiluminescence and exposed to film. These data reveal no significant differences in the expression of Bax (top panel) or Bcl-X (2nd panel). However, a large difference in Bcl-2 expression in MCF-7/EZ versus MCF-7/TREZ clones is identified (3rd panel). The TREZ clones express high levels of Bcl-2 protein while the EZ clones express nearly undetectable amounts. Overexposure of the anti-Bcl-2 blot (bottom panel) allows detection of the very low Bcl-2 expression in the TGF-β responsive MCF-7/EZ clones.

The top two panels in figure 6 reveal that there is no significant differences in the expression levels of Bax and Bcl-X, respectively. An additional western blot detected no difference in the expression level of Mcl-1 (data not shown). However, this is not the case for Bcl-2. Figure 6 (3rd panel) shows that there is a drastic difference in the amount of Bcl-2 protein expressed in the MCF-7/TREZ clones when compared to the MCF-7/EZ clones. All of the TGF-β nonresponsive MCF-7/TREZ clones express high levels of Bcl-2 while the TGF-β responsive MCF-7/EZ clones express very low, almost undetectable levels of Bcl-2.

These data suggest that autocrine TGF-β signaling in MCF-7 cells downregulates Bcl-2 expression and loss of a functional TGF-β autocrine loop results in high levels of Bcl-2 expression, as is seen in the MCF-7/TREZ clones. Furthermore, these data correlate with the differences observed in TNF-α induced cell death (Fig. 3) and suggest that the high level of Bcl-2 expression resulting from the loss of a functional TGF-β signaling cascade in the MCF-7/TREZ clones increases the cell's resistance to TNF-α induced cell death.

Our plans for Year 5 are as follows:

- Measurement of apoptosis-related phenomena following TNF treatment to compare with measurements of diminished total cell proliferation, again in TGF-β-responsive vs. TGF-β-nonresponsive MCF-7 cells. Thus far, we have followed overall cell number after 5 d incubation with TNF. We do not observe marked cell death with 24 h of adding TNF to the MCF-7/EZ cells. We will attempt to detect and quantitate apoptosis-related processes in these cells, over time. Possibilities are PARP cleavage, caspase activation, cytochrome C release, and overall morphology.

- Re-conversion of the TGF-β-nonresponsive MCF-7 cells into TGF-β-responsive cells by re-introduction of functioning TGF-β receptor signaling, to confirm that susceptibility to TNF is directly related to TGF-β responsiveness. Restoration of TGF-β signaling in the MCF-7/TREZ cells should result in a reduction in Bcl-2 expression and sensitization to TNF if our hypothesis is correct.

- Examination of the possible role of p53 in TGF-β-dependent TNF-mediated killing of MCF-7 cells. There have been reports of effects of TGF-β on p53 expression, and involvement of p53 in expression of Bcl-2. We will therefore evaluate p53 expression levels in our clones (Note: MCF-7 cells have wild-type p53).

Objective #2

This overall goal of this Objective is to evaluate the in vivo tumorigenic capacity of breast cancer cells with altered expression levels of TGF-β and/or diminished responsiveness to TGF-β (see pg. 3 of the site-visit report). In last year's report, we provided data from an experiment in which nude mice were injected with subclones of the MDA-MB231 human breast cancer cell line which differed in terms of TGF-β

production or responsiveness. We noted considerable heterogeneity in the rate of tumor-take within a clone, and an inadequate number of control cell injected animals with primary tumors to make comparison of metastatic rate reliable.

In reviewing the "history" of the clones we were comparing, realizing that the TGF-β-nonresponsive clones ('TREZ' clones) were generated over a year after the overexpressing clones ('C2S2' clones), we felt that the MDA-MB231 cell line, on continuous culture, might well had changed over that time period as a consequence of genetic drift. We therefore selected a C2S2 clone, and pCEN control clone and performed stable transfection with either the TREZ construct (described previously) or it's EZ control plasmid, selecting for hygromycin resistance. Just as described above, we determined that the TREZ clones were in fact non-responsive to TGF-β. These clones were then used in the next nude mouse experiment.

Since one of our goals is to compare the metastatic rate of the different clones, we needed to modify our experimental protocol to increase the rate of tumor take (you can't evaluate for metastasis unless the primary cancer developed). For this purpose, we injected each clone of cells with and without Matrigel. Matrigel, a gel-forming liquid derived from basement membrane and chock-full of extracellular matrix components, has been shown to augment the tumor take of a wide spectrum of human breast cancer cells upon sub-q injection into nude mice.

As before, female nude mice were injected with the cells, as described in Figure 7. Tumor take was more consistent this time. All animals injected with cells mixed with Matrigel developed tumor nodules. Two of the 6 animals in the pCEN-EZ and pCEN-TREZ groups did not develop grossly-evident tumors without Matrigel. Otherwise, all injected animals developed tumors. After 5 weeks, animals were killed and the tumor nodules excised. By gross observation, differences between p-CEN clones and C2S2 clones was evident. First of all, the tumors derived from C2C2 cells were larger, frequently had necrotic centers, and were overall more red-tinged in color upon cross-section. In contrast, the p-CEN tumors were smaller, pearly white in color throughout, and without necrotic centers. Abrogation of the TGF-β autocrine loop in the C2S2 cells (by the double transfection with TREZ) diminished the growth potential of these cells. These observations are consistent with our prior experiments. New to this experiment was the inclusion of Matrigel. We found that all clones demonstrated enhanced growth when the injection inoculum was mixed with Matrigel. From this, we can conclude that the augmentation in tumor growth by Matrigel is not mediated by TGF-β since it is not dependent upon the TGF-β responsiveness of the cells.

Since tumor take was more consistent in this experiment, we processed from each animal random sections of liver and lung for histochemical analysis. We also isolated genomic DNA from these tissues (as well as the primary tumor itself). These specimens will now be evaluated for the assessment and quantitation of metastatic spread.

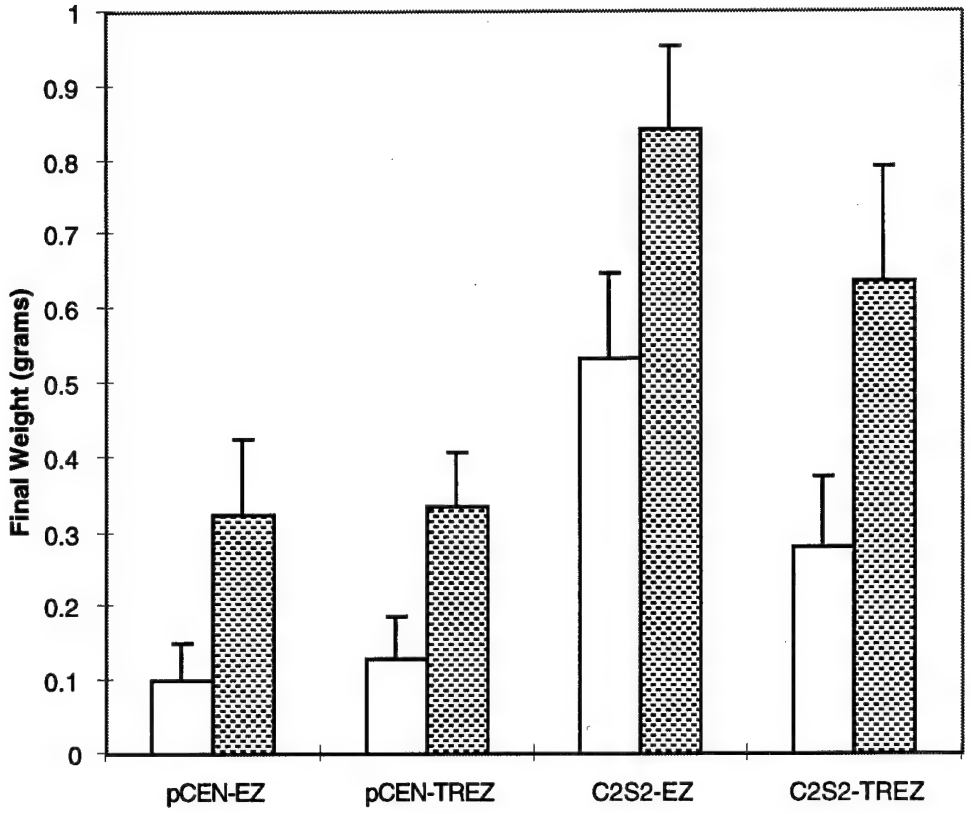

Figure 7. Tumor growth of MDA-MB231 cells in nude mice is altered by expression of and responsiveness to TGF-β and enhanced by Matrigel. Mice were injected sub-cutaneously with 3 X 10^6 tumor cells in 200 µl of serum-free MEM medium or 200 µl of a 50:50 mixture of serum-free medium and Matrigel. After 5 weeks, animals were sacrificed, tumors excised and weighed. There were 6 animals for each clone.

Our plans for this objective in year 5 are as follows:

- Continue with in vivo mouse experiments (at least one confirmatory experiment of results obtained in the prior year).
- Evaluate the metastatic burden by histologic examination of tissue specimens and PCR analysis of genomic DNA from liver and lung.

Objective #3

This objective deals with an observation we have previously reported regarding the promoter usage for TGF-β3. Specifically, we have observed that a downstream promoter (P2) is activated in breast cancer cells, but not in any other cell line thus far examined (Arrick et al., 1994). We have been attempting to understand the molecular basis for this difference. As described on pages 5 and 6 of the site visit report, this Objective initially included 3 sub-aims, the third of which has begun to yield

interesting positive data in year 4. It was recommended by the site visit panel that this aspect of Objective #3 constitute the focus of efforts in year 5.

In last year's report, we indicated that comparisons of CpG methylation status between two breast cancer cell lines (SK-BR3 and T47-D) and a non breast cancer cell line (HT-1080) demonstrated a marked difference in a handful of CpG sites closest to the transcription initiation site of P2 (methylated in the HT-1080 cells, not in the breast cancer cells). Our main accomplishment in year 4 with regard to this objective was an expanded analysis of TGF-β3 promoter methylation to include more of the CpG dinucleotides downstream of the breast cancer-specific promoter (P2), extending to the end of the first exon, and expanding our panel of cell lines to include two additional non breast cancer cell lines (A375 and A673).

We have also slightly altered our bisulfite treatment and subsequent PCR conditions to improve efficient targeting of this region of DNA. Genomic DNA from our panel of 5 cells lines was isolated and quantitated. Genomic DNA (20 µg) was digested with 40 Units *Pst*I at 37°C overnight. The digestion was spiked with an additional 20 U of *Pst*I followed by 3 additional hours at 37°C. DNA was purified using buffer saturated phenol/24:1 chloroform:isoamyl extraction followed by ethanol precipitation and resuspension in Tris Buffer/EDTA (TE). DNA was quantified and its purity assessed by measuring absorbance at $A_{260/280}$. 5 µg of digested DNA in 90 µl of water was alkaline denatured by incubation at 37°C for 15 minutes with 1/10 volume of 3 M NaOH followed by treatment with sodium bisulfite solution (900 µl of 5 M sodium bisulfite in 0.25 M hydroquione at pH 5.0) for 4 hours at 55°C. DNA was first desalted using the DNA Wizard Clean-Up System (Promega) followed by addition of 1/10 volume 3 M NaOH and incubation at 37°C for 15 minutes to desulfonate uracils. This was followed by ethanol precipitation and resuspension of the DNA in 20 µl TE.

We used a nested PCR approach to amplify the specific region of interest: an initial PCR reaction was carried out and then an aliquot of that reaction was subjected to a second round of PCR with primers internal to those used in the first round. All PCR reactions were initiated with the addition of Taq polymerase after 30 seconds into the initial 95°C soak ("hotstart").

The PCR reactions to amplify the region of P2 (extending from +425 to + 1501 relative to P1) used the following primers and reaction conditions:

FIRST PCR ROUND PRIMERS:

Upper - 5' ATT TTA TAT TTT AGT TAA TGA AGA YGA GAG GT 3'
Lower - 5' AAC TCC CAA CTC CAA TTC AAA CCC TCC A 3'

NESTED PRIMERS:

Upper - 5' TTC GAG GAA GTG TAA ATA AAA GAG AAA GTA TG 3'
Lower - 5' CAA ACC CTC CAA CAC AAA CAC CCC AAC R 3'
Y = C or T.
R = G or A.

The initial PCR followed the following parameters: 1X Buffer, 2.5mM $MgCl_2$, .2µM dNTPs, .5µM each primer, 5 U Taq DNA polymerase (Perkin Elmer) and 4µl bisulfite treated genomic DNA. This was taken to a final volume of 40µl with water. Reaction conditions were: 4 min (95°C soak), then [95°C (1 min), 50°C (1 Min), 72°C (3 min)] for 38 cycles. The nested PCR followed the following parameters: 1X Buffer, 2.5mM $MgCl_2$, .2µM dNTPs, .5µM each primer, 5 U Taq DNA polymerase and 1µl of the initial PCR reaction. This was taken to a final volume of 40µl with water. Reaction conditions were: 4 min (95°C soak), then [95°C (1 min), 52°C (1 Min), 72°C (90 sec)] for 40 cycles. After PCR, 10µl of the nested PCR was fractionated on a 1% agarose gel and visualized with Ethidium Bromide to confirm the size and purity of the product.

PCR products were ligated using the pGEM-T Vector System (Promega) as per the manufacturer's instructions. Ligation was allowed to proceed overnight at 4°C. DH5α *E. coli* made competent by treatment with calcium chloride were transformed with the ligation reactions by heat shock and plated on LB plates with 50 µg/ml Ampicillin, 4 µl IPTG (isopropylthio-β-D-galactoside), and 40 µl X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside). Plates were incubated at 37°C overnight and white colonies were selected. Bacteria were then grown in 2 ml of liquid LB at 37°C overnight in a shaking incubator. Plasmid DNA was then harvested by non-boiling plasmid miniprep and the plasmid DNA was resuspended in 20 µl TE + RNase A. 2 µl samples of these minipreps were subjected to digestion either by *Pvu* II to confirm the presence of the expected insert. Colonies containing plasmids with appropriately sized inserts were then regrown overnight in LB in the shaking incubator. DNA was extracted using the Qiagen Plasmid Miniprep Kit as per the manufacturer's instructions. The isolated plasmid DNA was then quantified and its purity assayed by O/D at $A_{260/280}$.

DNA sequencing reactions contained 400ng plasmid DNA, 8µl of termination mix, 3.2 pmol of the forward or reverse sequencing primer and water to 20 µl. The termination mix containing Taq and flourescently labeled ddNTPs was purchased, along with the sequencing primers, from the Dartmouth College Molecular Biology CORE facility. Sequencing conditions were: 25 cycles at [95°C (30 sec), 50°C (15 sec), and 60°C (4 min)]. The PCR samples were then purified using Centriflex Gel Filtration Columns (Advanced Genetic Technologies Corp.) as per manufacturer's instructions. Samples were then dried under vacuum and sent to the CORE facility for sequencing by the ABI Prism Big Dye Terminator Cycle Sequencing System.

Sequences were aligned and compared to the unconverted genomic DNA sequence of TGF-β3 using the SeqEd Version 1.0.3 computer program (Applied Biosystems). CpG sites in the wild type sequence were retained if the site was methylated while unmethylated sites were converted to TpGs. In the event that the SeqEd program was unable to distinguish which nucleotide was at a specific position, sequences were examined using the Edit View: ABI Automated DNA Sequence Viewer computer program by Perkin Elmer. Direct examination of the fluorescence at each nucleotide allowed for the sequence determination at these sites. In all cases, the nucleotide with the strongest overall signal at a given site was determined to be located there.

A compilation of the resulting data is presented in Figure 8. Each CpG is represented by a circle on a stick (lollipop) with the degree to which the circle is blackened equal to the percentage of clones showing methylation at that site. Completely black circles represent completely methylated CpGs; open circles represent unmethylated CpGs. It is clear from Figure 8 that there is a marked difference in methylation at the CpG sites upstream of P2, extending down to (but not beyond) the first exon-intron border.

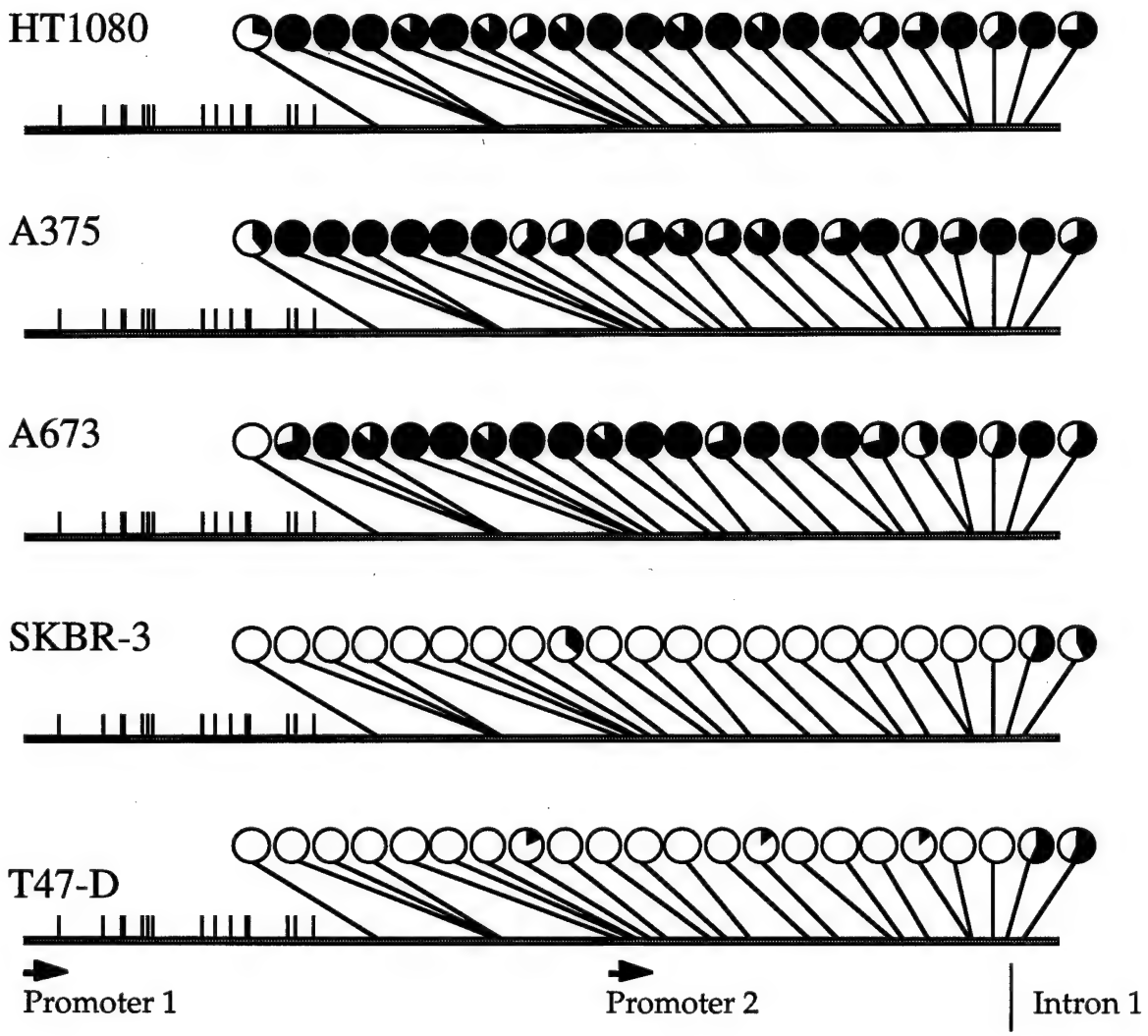

Figure 8. Methylation frequency at CpG sites flanking the breast-specific downstream promoter of TGF-β3. Each CpG is represented by a lollipop, the degree to which the circle is filled in is proportional to the percentage of clones showing methylation at that site. Completely black circles represent completely methylated CpGs; open circles represent unmethylated CpGs. Data from 4-8 independent determinations of methylation at each CpG site for genomic DNA from three non-breast cancer cell lines (HT1080, A375, and A673) and two breast cancer cell lines (SKBR-3 and T47-D) was used to generate this composite representation.

An overall summary of our findings with regard to CpG methylation at the TGF-β3 locus is provided in Figure 9. It is interesting to note that differences between breast and non breast cancer cells were restricted to a relatively small number of CpGs, essentially constituting all the CpGs within the first exon of the transcript generated by P2 and a small cluster of CpGs just upstream of P2's transcription initiation site. The heavy methylation at this portion of the CpG island is consistent with the shutting down of P2 in the non breast cancer cells.

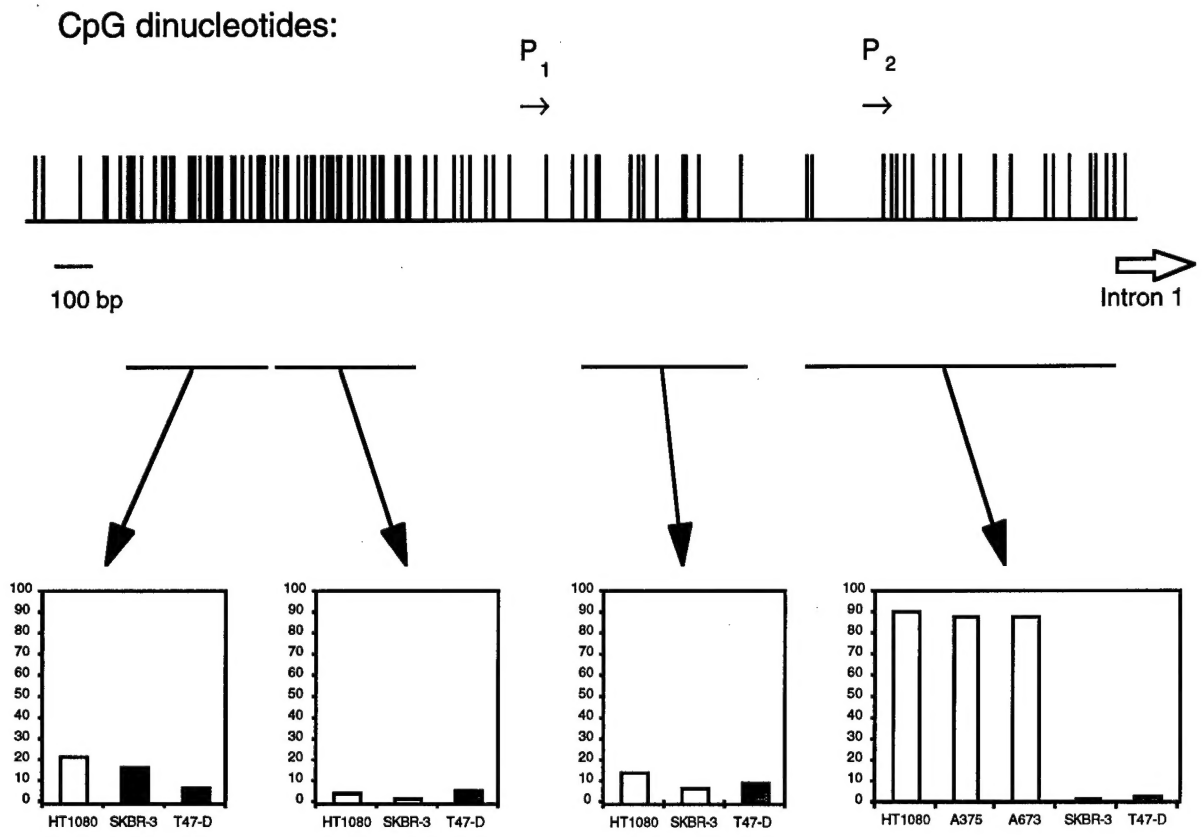

Figure 9. Overall methylation frequency within selected regions of the CpG island associated with TGF-β3. Within each of the indicated regions (5' half of the island core, 3' half of the island core, between P1 and P2, and lastly the P2-flanking region) the overall CpG methylation frequency (percentage) found for each cell line is plotted. Data from non-breast cancer cell lines is shown as open bars; bars with data from the breast cancer cell lines are filled.

Our plans for the completion of objective #3 in year 5 are as follows:

- Evaluate the effect of the demethylating agent azacytidine on TGF-β3 expression. There are examples of this form of "forced demethylation" resulting in the reactivation of promoters which had presumably been silenced by virtue of CpG

methylation. We will look to see if P2 becomes activated in non breast cancer cells following treatment with azacytidine.

• Evaluate the effect of the hormonal agent gestodene, which alters TGF-β3 promoter usage in T47-D cells, on CpG methylation status. If a change in CpG methylation coincides with this hormone's effect on TGF-β3 it would be further evidence that the observed methylation patterns are directly related to promoter usage, and might in the future serve as a model by which hormonal action can change chromatin structure.

Conclusions

Work related to objective #1 in the past year has focused upon the role of TGF-β in the cytotoxic effects of TNF-α. Our experiments have documented that, contrary to published work, TGF-β is playing a significant role as a determinant of cell susceptibility to TNF-α. We have also determined that TGF-β-mediated downregulation of Bcl-2 is the most likely explanation of our observations. A manuscript of this work is in progress.

We have not completed analysis of the tissues obtained from this past year's animal experiment (which only recently terminated), and cannot come to conclusions with regard to the role of TGF-β in the metastatic potential of these cells. We are consistently observing enhanced primary tumor growth in those cells which overexpress TGF-β.

Our detailed mapping of the methylation status of the TGF-β3 CpG island has revealed a marked difference at the 3' end of the island. Those cells not utilizing the downstream promoter (P2) showing nearly complete methylation, whereas the breast cancer cells, which do use this promoter, showed nearly complete unmethylation. Future experiments will be directed at determining whether there is a true cause-effect relationship here. For instance, we will determine if forced demethylation reactivate this promoter in non breast cancer cell lines.

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